Incidence of Extended Spectrum Beta-lactamase Producing Pseudomonas Aeruginosa and Frequency of OXA-2 and OXA-10 Genes

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Abstract: Pseudomonas aeruginosa has emerged as a major cause of infection in the last few decades. The objective of this study was to determine the distribution of extended spectrum beta-lactamase-producing P. aeruginosa and the frequency of OXA-2 and OXA-10 genes. 350 and 120 P. aeruginosa samples were isolated from patients in two locations in Iran representing the region of the Middle East. Antibiotic pattern and OXA type ESBLs were detected by disk diffusion and PCR methods. Of 350 isolates of P. aeruginosa isolates in the first location, 105 (30%) isolates were positive for ESBLs from which 92 (87.61%) isolates were positive for OXA-10 Type ESBLs while only 5 (4.76%) isolates were positive for OXA-2 ESBLs by using PCR methods in Ilam hospitals. In the second location, 46 (38.33%) of P. aeruginosa isolates were positive for ESBLs of which 29 (63%) were positive for OXA-10 Type ESBLs and only 1 (2.1%) isolates were positive for OXA-2 ESBLs by PCR methods in Kerman hospitals. Results of the current study showed the presence of high percentage of OXA genes, 2 and 10, ESBLs in the region of the Middle East associated with extended resistance profile against almost all cephalosporins. Moreover, comparing the results of the studied two cities indicated that distribution of OXA type ESBLs is locally diverse even in the same geographical region.

Key words: Pseudomonas aeruginosa, OXA-2, OXA-10, Gene, ESBLs

INTRODUCTION

The accelerated emergence of antibiotic resistance among the prevalent pathogens is the most serious threat to the management of infectious diseases. β-lactamase antibiotics are the most common treatment for bacterial infections (Blanc, et al 1998). Pseudomonas aeruginosa (P. aeruginosa) is responsible for 10–15% of nosocomial infections worldwide (Blanc, et al 1998). The infections are frequently difficult to treat because of both the natural resistance of the species and its remarkable ability to acquire resistance to multiple groups of antimicrobial agents. P. aeruginosa represents a phenomenon of antibiotic resistance, demonstrating practically all known enzymic and mutational mechanisms of bacterial resistance. These mechanisms are often present simultaneously, conferring combined resistance to many strains (McGowan, 2006).

Unfortunately, extended-spectrum β-lactamases (ESBLs) have been widely spread throughout serious infections of Gram-negative bacteria in the 1980’s (Bradford, 2001). The first report of plasmid-encoded β-lactamases capable of hydrolyzing the extended spectrum cephalosporins was published in 1983 (Khan, et al 1998). Hence, ESBLs are able to hydrolyze a broader spectrum of β-lactamases antibiotics than the simple parent β-lactamases from which they are derived. ESBLs have an ability to inactivate antibiotics containing an oxyimino-group such as oxyimino-cephalosporins (e.g., ceftazidime, ceftriaxone, cefotaxime) as well as oxyimino-monobactam (aztreonam) (Bradford, 2001); however, they are not active against cefamycins and carbapenems. Generally, they are inhibited by β-lactamase-inhibitors such as clavulanate and tazobactam (Yan, et al, 2006).

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P. aeruginosa has emerged as a major cause of infection in the last few decades. It is an increasingly prevalent opportunistic pathogen and is the fourth most frequently isolated nosocomial pathogen accounting for 9.9% of all hospitals acquired infections (Khan et al., 1998; Gordon et al., 1998). In burn units, P. aeruginosa outbreak leads to high mortality of up to 60% (Richard et al., 1994).

Various types of ESBLs have been identified in P. aeruginosa including class A ESBLs, such as TEM, SHV, PER and VEB ESBLs, and class D, such as OXA-type ESBLs. Among them, OXA-type ESBLs have been encountered most commonly in P. aeruginosa while class A ESBLs were found uncommon (Livermore, 2002, De Champs, 2002). Furthermore, most OXA-type ESBLs are OXA-2 and OXA-10 derivatives (Livermore, 2002).

The OXA-type ESBLs were originally discovered in P. aeruginosa isolates from a single hospital in Ankara, Turkey (Hall et al., 1993). Several OXA-type ESBLs have been derived from the original OXA-10 β-lactamase (e.g., OXA-11, 14, 6 and 17) (10-12). OXA-type β-lactamases are characterized by their high hydrolytic activity against oxacillin and cloxacillin and are poorly inhibited by clavulanic acid. Extension of the hydrolytic spectrum of oxacillinase to oxyimino cephalosporins has been reported in OXA-2 and OXA-10 extended-spectrum derivatives (13). In contrast to the majority of OXA-type ESBLs, which confer resistance to ceftazidime, the OXA-17 β-lactamase confers resistance against cefotaxime and ceftriaxone but provides only marginal protection against ceftazidime (Danel, et al., 1999). Many of the newer members of OXA-type ESBLs have been found mainly in P. aeruginosa isolates originating from Turkey and France (Hall, 1993; Philippon 1997). Despite the growing awareness of the impact of OXA-type ESBLs in the combat against bacterial infections, there have been few studies in different parts of the world conducted on the epidemiology and geographical spread of OXA-type ESBLs. This study was conducted to determine the distribution of extended spectrum β-lactamase producing P. aeruginosa and frequency of OXA-2 and OXA-10 genes in Kerman & Ilam hospitals.

MATERIALS AND METHODS

Bacterial Isolates:
A total of 350 and 120 P. aeruginosa samples were collected from Ilam and Kerman hospitals respectively between April 2007 to March 2008. Pseudomonas aeruginosa isolates were identified by using standard chemical methods (Pollack, 2000).

Detection of ESBLs in Pseudomonas aeruginosa:
Standard disk diffusion method – in vitro sensitivity testing- using established NCCLS procedure was carried out with cefpodoxime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), ceftriaxon (30 μg), amikacin (30 μg), cotrimoxazol (30 μg), imipenem (30 μg), and oxaciline (30 μg) (Hi Media, India). PCR-based detection of ESBLs Genes

Isolates were cultured in LB broth for 24 hours at 37°C and DNA was extracted by DNA extraction Kit (Fermentas, UE). The used primers for OXA-2 gene were: Forward 5-GCC AAA GGC ACG ATA GTT GT-3 and reverse 5-GCG TCC GAG TTG ACT GCC GG-3. This set of primers was used to amplify 700bp fragment of OXA-2 gene. On the other hand, the primers for OXA-10 were forward: 5-GT CTT TCG AGT ACG GCA TTA -3 and reverse 5-ATT TTC TTA GCG GCA ACT TAC-3. This set of primers was used to amplify a 720bp fragment of OXA-10 gene.

All used PCR reagents were from (Promega Corporation, Madison, USA). OXA-2 gene was amplified under the following condition: the initial denaturation was for 5 minutes at 94°C, followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 1 minute at 50°C, and extension for 1 minute at 72°C, and a final extension step for 10 minutes at 72°C. On the other hand, OXA-10 gene was amplified under the following condition: the initial denaturation was for 5 minutes at 94°C, followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 1 minute at 54°C, and extension for 1 minute 72°C, and a final extension step for 10 minutes at72°C (De Champs,2000,Vahaboglu, 1998). It is noteworthy to mention that negative control, master mix devoid of genomic DNA, and positive control, a strongly consistent positive sample, were used simultaneously in duplicates. PCR products, amplicons, were run in 1% agarose gel. The gels were stained with ethidium bromide (Sigma, USA) and a single band was observed at the desired position on ultraviolet light transilluminator (Vilber Lourmat, Cedex, France); bands were photographed using gel documentation system (Bio Rad Gel Doc 2000 Model Imaging System).
Many studies have investigated the prevalence of ESBLs in *P. aeruginosa*; according to these studies, a diverse range of ESBLs is currently emerging in hospitals (Weldhagen, 2003; Chanawong, 2001; Ben-Hamouda, 2004). Because of the large number of new β-lactamases, some with identical or very similar isoelectric points, described in *P. aeruginosa* in the past few years, isoelectric focusing can no longer be considered an effective method of identifying β-lactamases in this species. Therefore, epidemiological studies might better use PCR-based detection tests followed by analysis of PCR products by sequencing or restriction with endonucleases for detecting changes generated by point mutations. PCR–restriction fragment length polymorphism (RFLP) analysis has been successfully applied to the identification of TEM β-lactamases in Enterobacteriaceae (Arlet, 1995). In *P. aeruginosa*, PCR use has been limited to the detection of OXA-10-derived ESBLs without fully differentiating variants within this group (Vahaboglu, 1998). The main difficulty in applying PCR–RFLP to the characterization of oxacillinases is that they constitute a heterogeneous group of enzymes, including subgroups with large evolutionary distances between them (Sanschagrin, et al, 1995).

The results of the current study showed that 105 (30%) of 350 isolates of *P. aeruginosa* isolates were positive for OXA gene by screening disk diffusion methods (Table 1); Interestingly, by PCR methods, 92 (87.61%) isolates were positive for OXA-10 Type ESBLs while only 5 (4.76%) isolates were positive for OXA-2 ESBLs where two of them were positive for OXA-10 and OXA-2 at the same time. Ten (9.52%) isolates were negative for OXA genes by PCR (Figure 1).

### Table 1: Antimicrobial resistance pattern for *Pseudomonas aeruginosa* in Ilam hospitals

<table>
<thead>
<tr>
<th>Patients</th>
<th><em>P. aeruginosa</em> N (%)</th>
<th>Ceftazidim N (%)</th>
<th>Cefteriaxon N (%)</th>
<th>Oxacillin N (%)</th>
<th>Amikacin N (%)</th>
<th>Imipenem N (%)</th>
<th>Tikaricilin N (%)</th>
<th>Azteronam N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>210 (60)</td>
<td>160 (76.19)</td>
<td>126 (60)</td>
<td>68 (32.38)</td>
<td>150 (71.42)</td>
<td>12 (5.71)</td>
<td>82 (39.04)</td>
<td>86 (40.95)</td>
</tr>
<tr>
<td>Septum</td>
<td>87 (24.86)</td>
<td>54 (62.06)</td>
<td>38 (43.67)</td>
<td>26 (29.88)</td>
<td>48 (55.17)</td>
<td>5 (5.74)</td>
<td>43 (49.42)</td>
<td>33 (37.93)</td>
</tr>
<tr>
<td>Burn</td>
<td>53 (15.14)</td>
<td>21 (39.62)</td>
<td>18 (33.96)</td>
<td>11 (20.75)</td>
<td>35 (66.03)</td>
<td>2 (3.77)</td>
<td>29 (54.71)</td>
<td>14 (26.41)</td>
</tr>
<tr>
<td>Total</td>
<td>350 (100)</td>
<td>235 (67.14)</td>
<td>182 (52)</td>
<td>105 (30)</td>
<td>233 (66.5)</td>
<td>19 (5.4)</td>
<td>154 (44)</td>
<td>133 (38)</td>
</tr>
</tbody>
</table>

As far as our knowledge, this study presents the first report of isolation of *P. aeruginosa* isolates harbouring OXA-2 and 10 groups of ESBLs in the region of the Middle East. In this study, of 350 *P. aeruginosa* samples in Ilam Hospitals, 133 isolates were resistant to azteronam and 3rd generation of cephalosporin and suspect to Ambler’s class A ESBLS and 105 isolates were resistant to oxacillin and suspect to OXA type of ESBLs. Distribution of *P. aeruginosa* with OXA type gene from highest to lowest was in urine, sputum, and burn patients.

There is no phenotypic confirmatory method for the presence of OXA gene in *P. aeruginosa* (Kernodle, and Kaiser, 2000). Hence, the current study provides precise and the only reliable genotypic method for detecting OXA resistance genes in *P. aeruginosa*. In a similar study conducted by Bert et al. (Bert, et al, 2002), they reported that PCR detecting OXA-10 gene in *P. aeruginosa* isolates was positive in 68 (26.3%) isolates; 31 carried blaOXA-10, one carried blaOXA-14 and 36 carried a new variant intermediate between blaOXA-13 and blaOXA-19. Two other isolates carried blaOXA-2 variants encoding ESBLs differing from OXA-2 by a single amino acid substitution (Asp150 to Tyr and Trp159 to Cys, respectively).

In the current study, resistance *P. aeruginosa* isolates against ceftazidim, cefteriaxon, tikaricilin, amikacin, and azteronam were seen along with oxacillin but, the distribution of resistant *P. aeruginosa* against imipenem was minimal. The results of the current study were found to be congruous with that of previous studies. The resistance pattern observed in other studies for *P. aeruginosa* ED-1 resembled that of OXA-15, an extended-spectrum derivative of OXA-2 (Danel et al, 1997). And it was stated that most of the extended-spectrum oxacillinases confer resistance to ceftazidime and cefotaxime which are not inhibited significantly by clavulanic acid (Danel, et al, 1998; Danel, 1995; Mugnier, 1998). Cefazidime resistance in *P. aeruginosa* is most often caused by hyperproduction of the chromosomal class C cephalosporinase or by efflux or impermeability; these mechanisms raise the MICs of ureidopenicillins and cefotaxime above that of ceftazidime (Chen, et al, 1995).

Levels of antimicrobial susceptibility and mechanisms of resistance to antipseudomonal agents of *P. aeruginosa* were evaluated by a European team, Strateva et al., (2007) who showed close results to our study except for Imipenem. They surveyed 203 clinical isolates and reported that the antibiotic resistance rates against the following antimicrobials were: carbenicillin 93.1%, azlocillin 91.6%, piperacillin 86.2%, piperacillin/tazobactam 56.8%, cefazidime 45.8%, ceftipime 48.9%, cefpirome 58.2%, aztreonam 49.8%, and piperacillin/tazobactam 56.8%.

### Results and Discussion


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*Table 1: Antimicrobial resistance pattern for *Pseudomonas aeruginosa* in Ilam hospitals*

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**As far as our knowledge, this study presents the first report of isolation of *P. aeruginosa* isolates harbouring OXA-2 and 10 groups of ESBLs in the region of the Middle East. In this study, of 350 *P. aeruginosa* samples in Ilam Hospitals, 133 isolates were resistant to azteronam and 3rd generation of cephalosporin and suspect to Ambler’s class A ESBLS and 105 isolates were resistant to oxacillin and suspect to OXA type of ESBLs. Distribution of *P. aeruginosa* with OXA type gene from highest to lowest was in urine, sputum, and burn patients.**
imipenem 42.3%, meropenem 45.5%, amikacin 59.1%, gentamicin 79.7%, tobramycin 89.6%, netilmicin 69.6% and ciprofloxacin 80.3%. They reported that structural genes encoding class A and class D β-lactamases were as follows: blaVEB-1 33.1%, blapSE-1 22.5%, blaPER-1 0%, blaOXA-groupI 41.3% and blaOXA-groupII 8.8%. This indicates that the genetic pool of P. aeruginosa in Europe and in the Middle East are highly alike. Therefore, the pattern of antimicrobial resistance in either region can be of high predictability for the other region. Nevertheless, resistance against imipenem was found minimal in the region of the Middle East when compared to that observed in Europe. Taken that resistance against imipenem is a serious problem for treating patients with P. aeruginosa infection, the overall effectiveness of antipseudomonal agents in the Middle East might be better than that in Europe.

In Kerman hospitals, of 120 P. aeruginosa isolates 46 (38.33%) isolates were positive for OXA gene by disk diffusion method (Table 2). Of 46 OXA-positive P. aeruginosa isolates, by PCR methods, 29 (63%) were positive for OXA-10 Type ESBLs and 1 (2.1%) isolate was positive for OXA-2 ESBLs which was positive to both OXA-2 and OXA-10 (Figure 1).

Table 2: Antimicrobial resistance pattern for Pseudomonas aeruginosa in Kerman hospitals

<table>
<thead>
<tr>
<th>patients</th>
<th>ceftazidim</th>
<th>cefteriaxon</th>
<th>oxacilin</th>
<th>amikacin</th>
<th>imipenem</th>
<th>Tikaricilin</th>
<th>azteronam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burn</td>
<td>78(65%)</td>
<td>54(45%)</td>
<td>46(38.33)</td>
<td>69(57.5%)</td>
<td>-</td>
<td>65(54.1%)</td>
<td>72(60%)</td>
</tr>
</tbody>
</table>

Fig. 1: Gel electrogram of PCR products of isolated P. aeruginosa with positive OXA-10 gene, 720 bp, (lanes 1-5) and OXA-2 gene, 700 bp, (lanes 7-9). (Lane 6) is the negative control and (M) is the molecular weight ladder (scale 50bp).

Interestingly, in Kerman hospitals, it was found that there was no resistance of P. aeruginosa isolates against imipenem. The results also showed a remarkable high resistance to azteronam, 60%, which determines an ESBL type of P. aeruginosa in Kerman different from that in Ilam using the disk diffusion method. This provided evidence that the antimicrobial resistance of P. aeruginosa can be finely diverse even in the closely related geographical regions. However, in both Kerman and Ilam, the rate of P. aeruginosa isolates with OXA-10 was high and close to each other. This indicates that Oxacillin resistance of P. aeruginosa in the Middle East region seems to be essential. Nevertheless, in a previous survey on burn patients in Tehran hospitals in 2007-2008 (Mirsalehian, et al 2008), only 29% of isolates were OXA-10 positive which is far lower than that found in the current study. This can be explained that OXA genes are acquired more quickly than other genes and spread progressively in the population of P. aeruginosa bacteria. Therefore, this pinpoints to the importance of conducting frequent screenings of antimicrobial resistance of P. aeruginosa and conducting particularly frequent genotypic screenings on OXA genes in P. aeruginosa. Similar to Ilam hospitals, P. aeruginosa isolates in Kerman showed very clear extended spectrum of cephalosporins resistance which strengthening the proposed correlation between OXA genes infiltration in P. aeruginosa population and the extended-spectrum cephalosporins resistance.

Conclusions:
In conclusion, this study highlighted the contribution of the OXA-2 and 10 ESBLs to the extended-spectrum cephalosporins resistance in P. aeruginosa of the Middle East region. Moreover, this study identified an OXA-2 and 10 producing strain of P. aeruginosa for the first time in the Middle East region. Early detection of these isolates and conducting frequent screenings could acquire good information concerning their
dissemination as outbreaks caused by \textit{P. aeruginosa} are usually difficult to control. Finally, \textit{P. aeruginosa} may, like the Enterobacteriaceae, constitute a reservoir of ESBL genes. The spread of \textit{P. aeruginosa} strains producing OXA-2 and 10 may be enhanced by under detection.

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**REFERENCES**


